

Applicants: Philip O. Livingston and Friedhelm Helling
Serial No.: 08/475,784
Filed: June 7, 1995
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Please amend the subject application as follows:

Amendments to the Specification:

Please replace the paragraph beginning at page 65, line 30, with the following amended paragraph:

Immunological adjuvants: DETOX was produced and supplied by Ribi Immunochem Research Inc. (Hamilton, MT), formulated as a lyophilized oil droplet emulsion. It consists of cell wall skeletons (CWS) from bacille Calmette-Guorin and monophosphoryl lipid A (MPLA) from Salmonella minnesota R595. On the day of vaccination, 0.25 ml DETOX (250 μ g CWS and 25 μ g MPLA) was mixed with the GM2-KLH preparation. The vaccine (final volume 0.75ml) was vortexed for 2-3 minutes and administered to the patients within 15 min. BCG was purchased from Bionetics Research Inc. (Rockville, MD). On the day of vaccination, 107 viable units of BCG in 0.1 ml normal saline were added to the GM2-KLH vaccine in each individual syringe (final volume 0.6 ml). The contents were mixed and administered to the patients within 15 min. QS-21 adjuvant (a homogeneous saponin purified from the bark of Quillaja saponaria Molina (10, 11) was kindly provided by Cambridge Biotech Inc. (Worcester, MA). To obtain QS-21, the procedure of Kensil et al, 1991 (10) may be employed. Specifically, coarsely chopped Q. saponaria bark (approximately 1 cm square, obtained from Hauser Chemicals, Boulder, CO) was stirred with 10 ml of water/g of bark at room temperature for 1 h. The

extract was centrifuged and the supernatant containing the solubilized saponins was saved. The extraction step was repeated on the bark pellet and the two supernatants were pooled. To remove nonsaponin components, the supernatant pool was lyophilized, redissolved in 40 mM acetic acid in water at a concentration of 250 mg/ml (w:v) and either chromatographed through Sephadex G-50 (medium, Pharmacia, Piscataway, NJ) in 40 mM acetic acid with the hemolytic activity localized in the void volume fraction, or dialyzed against 40 mM acetic acid with the hemolytic activity retained by the dialysis membrane. The hemolytic fraction was lyophilized and redissolved at a concentration of 200 mg/ml in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v); 1 g of this fraction was applied to Silica Lichroprep (E.M. Science, Gibbstion, NJ; 40 to 63 μ M particle size, 2.5 cm I.D. x 20 cm height) and eluted isocratically in the solvent used to solubilize the saponins. The elution of saponins was monitored by carbohydrate assay. Fractions containing the saponins of interest were identified by reverse phase TLC with visualization with Bial's reagent (Sigma, St. Louis, MO) pooled individually, and rotavapped to dryness. The fractions from the silica chromatography were then redissolved in 40 mM acetic acid in 50% methanol and loaded on a semipreparative HPLC column (Vydac C₄, 5 μ m particle size, 3000 nm pore size, 10 mm I.D. X 25 cm length). Saponin peaks, detected by absorbance at 214 nm, were eluted by using a methanol gradient at a flow rate of 4 ml/min, and

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individually rotavapped to dryness. Purity of
saponins was assessed by analytic HPLC (Vydac C₄,
5μm particle size, 3000 nm pore size, 4.6 mm I.D. X
25 cm length) with a gradient of 0.1% TFA in
acetonitrile. As described in Newman et al., 1992
(11), QS-21 is defined as the adjuvant active
reverse phase HPLC fraction 21 from Q. Saponaria
bark extract. 100μg or 200 μg of QS-21 were diluted
in 0.25ml normal saline and mixed with GM2-KLH. The
vaccine (final volume 0.75ml) was vortexed for 2-3
minutes and administered within 15 min.